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MODIFICATION OF TUBULIN WITH THE FLUOROCHROME 5-(4,6-DICHLOROTRIAZIN-2-YL)AMINO FLUORESCEIN AND THE INTERACTION OF THE FLUORESCENT PROTEIN WITH THE ISOLATED MEIOTIC APPARATUS

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Abstract

Microtubules (MTs) assembled *in vitro* have been labeled with the fluorochrome 5-(4,6-dichlorotriazin-2-yl)amino fluorescein (DTAF) using a modification of the procedure of Keith *et al.* (1981). When the fluorescent MTs were analyzed by polyacrylamide gel electrophoresis under reducing conditions both the major components of the MTs, the tubulin dimers and microtubule-associated proteins (MAPs), appeared distinctly fluorescent and thus were modified covalently with the fluorochrome. The fluorescent MTs were cold labile and reassembled with kinetics identical to control microtubule proteins. In addition, fluorescent tubulin dimers, purified from the mixture of labeled MT proteins, assembled with both an initial rate and final extent of assembly indistinguishable from a control, unlabeled sample when both were examined at the same protein concentration. On occasion, however, the fluorescent protein has been observed to demonstrate a one to two minute lag, an anomaly that is a function of total protein concentration. When the reassembled fluorescent MTs were examined by negative stain electron microscopy, morphologically normal polymers were observed. Since fluorescent tubulin dimers are potentially valuable probes for the examination of MT growth polarity, the ability of the fluorescent protein to assemble in association with nucleation centers *in vitro* was examined. For these experiments, meiotic spindles from the surf clam *Spisula solidissima* were isolated and used to nucleate the assembly of fluorescent MTs *in vitro*. The results of such experiments revealed linear, fluorescent arrays within the spindle and asters. The results of the experiments reported here demonstrate that tubulin labeled with DTAF retained its ability to self-assemble and to assemble in association with microtubule organizing centers (MTOCs) *in vitro*. Moreover, the polymer formed from the DTAF labeled proteins was distinctly fluorescent and could be observed and recorded using conventional fluorescence optics.

Introduction

Much of our knowledge of the cytoskeleton has been obtained from conventional electron microscopy and immunocytochemical techniques. Ultrastructural analyses (Porter, 1966; Tilney, 1971; Willingham *et al.*, 1981) revealed details of the structural organization of the cytoplasm, while immunocytochemistry, performed using antibodies specific for particular cytoskeletal proteins, illustrated the distributions of, and spatial relationships among, these components (Lazarides, 1980; Ball and Singer,

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1981; Herman *et al.*, 1981). However, because these techniques require fixed preparations, rearrangements of cytoskeletal proteins during motile events cannot be determined directly.

To investigate the molecular dynamics of the cytoskeleton in living cells, Taylor and Wang (1978) introduced the technique of Molecular Cytochemistry, more recently termed Fluorescent Analog Cytochemistry (FAC, Wang *et al.*, 1982). In this technique, based on the initial work of Sanger (1975), fluorescent derivatives of cellular proteins which retain their native characteristics were prepared and introduced into living cells; the cells were then observed with a fluorescence microscope. This technique, which has been used to study a variety of different cytoskeletal components (Taylor and Wang, 1978; Feramisco, 1979; Kreis *et al.*, 1979; Burridge and Feramisco, 1980; Gawlitta *et al.*, 1980; Keith *et al.*, 1981; Glacy, 1983), has revealed the incorporation of the fluorescent derivatives into cellular structures. More importantly, the distribution and the changing pattern of the fluorescent protein can be monitored in living cells during various motile events. This report describes a modification of the procedure of Keith *et al.* (1981) for the preparation of tubulin modified with the fluorochrome 5-(4,6-dichlorotriazin-2-yl)amino fluorescein (DTAF). The procedure is rapid and yields modified, fluorescent tubulin in quantitatively reproducible amounts, a result not achieved using previously published procedures. The modified protein probe retains its native characteristics as judged by a number of criteria; moreover the fluorescent derivative incorporated into MTs of isolated spindles which could be observed using conventional fluorescence microscopy.

Materials and Methods

Protein purification

Microtubule proteins (MTP) were purified from pig brains using cycles of temperature-dependent assembly and disassembly as described by Sloboda *et al.* (1976). Polymerization buffer (PM) consisting of 0.1 *M* Pipes, pH 6.9; 1 m*M* $MgSO_4$; 2 m*M* EGTA; and 1 m*M* GTP was used throughout the procedure. Brain tissue was homogenized using a glass teflon homogenizer with a motor driven pestle in 0.5 ml of buffer per gram of tissue. The supernatant obtained after centrifugation of the brain homogenate for 1 hour at 100,000 × *g* was mixed with an equal volume of PM buffer containing 8 *M* glycerol (PMG). MTs, assembled during incubation at 37°C for 40 min, were collected by centrifugation at 100,000 × *g* for 40 min, resuspended in fresh PM using a dounce homogenizer, and depolymerized on ice for 30 minutes. The depolymerized MTs were clarified by centrifugation at 100,000 × *g* for 30 min. The supernatant was again mixed with an equal volume of PMG, polymerized, and spun as before. The sedimented MTs were overlaid with PM containing 4 *M* glycerol and stored at −70°C until use. Tubulin dimers were prepared free of MAPs using phosphocellulose column chromatography (Weingarten *et al.*, 1975; Williams and Detrich, 1979; Sloboda and Rosenbaum, 1982). Column buffer consisting of 50 m*M* Pipes, pH 6.9; 0.5 m*M* $MgSO_4$; 1.0 m*M* EGTA; and 0.1 m*M* GTP was used throughout the procedure.

When required, fluorescent MAPs were prepared using a modification of the taxol procedure of Vallee (1982). Briefly, three times cycled MTs were polymerized in PM buffer containing 20 μM taxol (PMT). The MTs were then labeled with the fluorochrome DTAF for 5 min at 37°C (see Results) and collected by centrifugation at 100,000 × *g* for 30 min. The fluorescent MTs were resuspended and washed once in PMT. The salt concentration of the resuspended MTs was then adjusted to 0.35 *M* using a concentrated solution of NaCl. The fluorescent MTs, which had been

stripped of MAPs by the salt, were separated from the solubilized MAPs by centrifugation at 30,000 × *g* for 30 min. The fluorescent MAPs were recovered from the supernatant and desalted on Sephadex G-25 using centrifuge desalting columns (Neal and Florini, 1973).

Electron microscopy

Diluted solutions of microtubules were applied to formvar coated copper grids, allowed to adsorb for approximately 1 min, and stained with millipore filtered aqueous uranyl acetate and air dried. Grids were examined in a JEOL 100CX electron microscope operated at 60kV.

Meiotic spindle isolation

Meiotic spindles were isolated from activated *Spisula solidissima* oocytes as described by Murphy (1980) with several minor changes. Ooctyes, activated by raising the potassium concentration of the sea water by 38 m*M* using a 0.52 *M* KCl solution (Allen, 1954), were sedimented in a hand centrifuge, washed twice in 1 *M* glycerol, 1 m*M* Tris, pH 8.0, and 2 m*M* EGTA, and resuspended in Isolation Medium (IM) consisting of 10 m*M* MES, pH 6.2; 5 m*M* EGTA; 1.0% Nonidet NP-40; 0.5 *M* glycerol, and 0.5 m*M* $MgSO_4$ at room temperature. The eggs were lysed by shaking and the isolated meiotic spindles were collected by centrifugation for 5 min at 3000 × *g* in a clinical centrifuge.

Microscopy

Phase contrast, fluorescence, and polarized light microscopy were performed using a Zeiss Photomicroscope III available through the generous loan of the Carl Zeiss Co. to the students and staff of the Physiology Course, Marine Biological Laboratory, Woods Hole, Massachusetts. For phase contrast and fluorescence observations, a Zeiss 63× planapochromat lens (N.A. = 1.4) was used. A 10× pol objective and Brace-Kohler compensator were used for polarized light observations.

Other methods

SDS polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970) using a 5–20% gradient of acrylamide. Proteins were stained with Coomassie Brilliant Blue R according to Fairbanks *et al.*, (1971). Unstained gels were fixed in 10% acetic acid, washed in 50 m*M* Tris, 150 m*M* NaCl, 0.02% NaN_3, pH 7.5 (Burridge, 1976), and photographed under long wave-length ultraviolet illumination using an Edna Lite 606 filter and Tri-X film. Turbidity measurements (Gaskin *et al.*, 1974) were performed using a Gilford model 250 recording spectrophotometer fitted with a thermal cuvette holder. Fluorescence measurements were made using a Turner model 430 spectrofluorometer equipped with an Aminco photon counter and a 150 watt Xenon lamp. Protein concentration was determined by the method of Lowry *et al.* (1951) as modified by Schacterle and Pollack (1973) using a BSA standard.

Results

Fluorescent labeling of microtubules

MTs in the assembled state were labeled with fluorochrome so that sites on the tubulin dimers critical for the polymerization of a MT would be protected from

reaction with the fluorochrome. The fluorescent labeling reagent 5-(4,6-dichlorotriazin-2-yl)amino fluorescein (DTAF), which reacts covalently with ϵ-amino groups of lysine residues and N-terminal amino groups (Blakeslee and Baines, 1976), was mixed with the polymerized MTs at 37°C. No decrease in turbidity, which would indicate MT disassembly, was observed during the labeling reaction. The fluorochrome, prepared fresh daily in dimethyl-sulfoxide, and assembled microtubule proteins were combined using a 2–4 fold molar excess of fluorochrome to protein; the labeling reaction was performed for 5 min (see Keith *et al.*, 1981). Immediately after labeling centrifugation was begun to collect the fluorescent polymer and separate the MTs from the bulk of the fluorochrome, thereby terminating the labeling reaction. The fluorescent MT pellets were resuspended in fresh PM and depolymerized on ice. To ensure that any remaining unbound fluorochrome was removed from solution, the depolymerized proteins were desalted using Sephadex G-25 in centrifuge columns (Neal and Florini, 1973). The fluorescent proteins were then subjected to a further round of temperature-dependent assembly and disassembly to select for assembly competent proteins. Purified fluorescent tubulin dimers were prepared using phosphocellulose column chromatography (see Methods). Thus, the major modifications of the technique of Keith *et al.* (1981) reported here are (i) a decrease in fluorochrome to protein ratio during labeling, (ii) a decrease in labeling time, and the addition of (iii) a centrifugation step, (iv) a gel chromatography step, and (v) a further round of assembly and disassembly prior to purification of the labeled dimers by phosphocellulose chromatography.

Characteristics of the fluorescent proteins

When the fluorescent MT proteins were analyzed by polyacrylamide gel electrophoresis both the α- and β-tubulins and high molecular weight microtubule-associated proteins (MAPs, Sloboda *et al.*, 1975) were distinctly fluorescent. In addition, after purification of the tubulin dimers by phosphocellulose chromatography and the MAPs by a taxol procedure (see Methods), both protein fractions remained distinctly fluorescent (Fig. 1); the fluorescence co-migrated with the protein and no fluorescent breakdown products were observed. Since these are denaturing gels run under reducing conditions (see Methods), these observations demonstrate that the fluorochrome is covalently bound to the protein.

Several experiments were performed to determine if the modified proteins retained their native characteristics. For example, the assembly kinetics of the fluorescent proteins were compared with control unlabeled proteins assembled at the same protein concentration. As seen in Figure 2, three times cycled, fluorescent microtubule proteins assembled with both an initial rate and final extent of assembly identical to the unlabeled proteins. Next, purified fluorescent tubulin dimers were prepared and tested to determine if they also retained the same assembly characteristics as unlabeled tubulin dimers after separation from the MAPs. As seen in Figure 3, the fluorescent tubulin dimers assembled with kinetics indistinguishable from the unlabeled control sample. The lag apparent in the experimental sample (F) in Figure 3 occurs occasionally at low protein concentrations (1–3 mg/ml). When examined using negative stain electron microscopy, MTs assembled from the fluorescent subunits were observed to be morphologically normal (Fig. 4).

In addition, the excitation and emission spectra of the fluorescent microtubule proteins were determined. The excitation and emission maxima were 495 nm and 520 nm, respectively (Fig. 5). The wavelengths of maximal excitation and maximal emission were identical using either polymerized or depolymerized MT proteins for the determination. These data suggest that polymerization does not alter these values within the resolution of the instrument used.

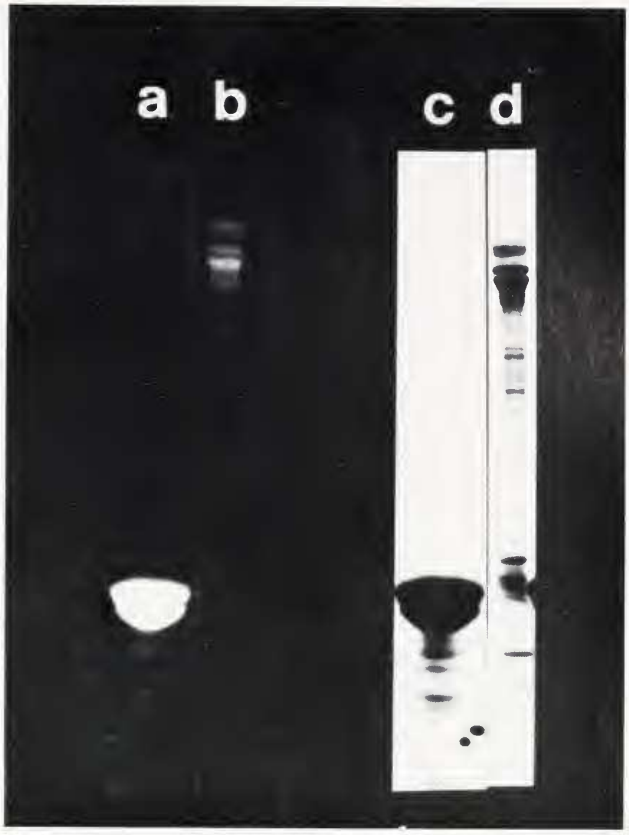

FIGURE 1. Polyacrylamide gel electrophoresis of the fluorescently modified proteins. The unstained gels were photographed under UV illumination to visualize the fluorescence (a and b); identical gels were then stained for protein with Coomassie Brilliant Blue (c and d). Lanes a and c are phosphocellulose purified DTAF labeled tubulin dimers; lanes b and d are DTAF labeled MAPs prepared according to Vallee (1982) (see Methods). Both the α- and β-subunits of tubulin are distinctly fluorescent as are the high molecular weight MAPs.

The stoichiometry of labeling was determined as described by Keith *et al.*, (1981) using an extinction coefficient of 5.4×10^5 for DTAF and a molecular weight of 110,000 for the tubulin dimer. At a final stoichiometry of 0.003–0.1 (moles of fluorochrome bound per mole of protein) the proteins retained their native characteristics and were distinctly fluorescent.

Interaction of the fluorescent protein with the isolated mitotic apparatus

To determine whether the fluorescent protein retained the ability to assemble in association with microtubule organizing centers (MTOCs, Pickett-Heaps, 1969) *in vitro,* the fluorescently modified protein was incubated with meiotic spindles isolated from oocytes of the surf clam, *Spisula solidissima.* Incubation of isolated spindles with fluorescent MT protein maintained or augmented the birefringence of the isolated spindles in a temperature and concentration dependent fashion (Fig. 6). For example, when isolated *Spisula* meiotic apparatuses (MAs) (Fig. 6a) were resuspended in po-

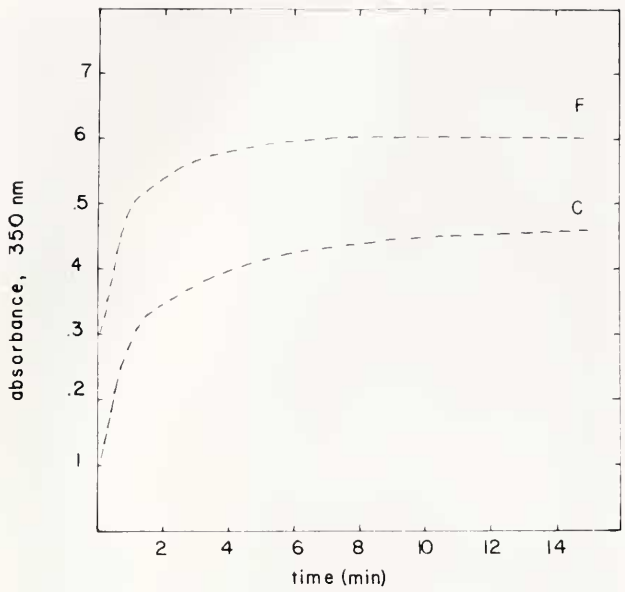

FIGURE 2. Kinetics of assembly of a DTAF labeled (F) and control (C) sample of microtubule proteins, each at 2.0 mg/ml. Assembly was monitored at 37°C using a temperature controlled cuvette holder. Apparent absorbance at 350 nm has been plotted as a function of time; the initial A_{350} values have been offset along the ordinate using the controls on the spectrophotometer. Note that the total change in A_{350}, indicative of the total mass of assembled polymer, is the same for both samples, as is the initial rate of assembly.

lymerization buffer (PM) and placed on ice, the spindle MTs disassembled and only very faint birefringence (BR) remained (Fig. 6b). However, when aliquots of the same preparation of spindles (Fig. 6a) were resuspended in PM buffer containing MT proteins, chilled, and rewarmed, spindle BR was observed in proportion to the concentration of MT proteins present (Fig. 6c, d).

When the isolated spindles, which had been resuspended in solutions containing fluorescent microtubule proteins, were examined using fluorescence optics, distinctly fluorescent spindles with linear, fluorescent fibers emanating from the spindle poles were seen (Fig. 7a, b). This result suggests that the fluorescent proteins were incorporated into the spindle and astral regions of the MA, an observation consistent with previous experiments performed using unlabeled brain MT proteins to augment the BR of meiotic (Inoué *et al.*, 1974; Rebhun *et al.*, 1974) or mitotic (Cande *et al.*, 1974) spindles. When calcium was added to the fluorescent spindles, a rapid change in their appearance was observed (Fig. 7c, d). The linear fluorescent fibers were reduced to amorphous fluorescence (Fig. 7d). Similarly, the linear appearance of the spindle fibers observed in phase contrast was abolished (Fig. 7c). This sensitivity of the fluorescence to millimolar concentrations of calcium suggests that the fluorescent pattern shown in Figure 7b was due to the polymerization of calcium labile, fluorescent MTs.

DISCUSSION

The results presented here demonstrate that MTs can be modified with the fluorochrome DTAF as previously reported in Keith *et al.* (1981). The modification of the microtubule proteins with the fluorochrome is covalent, as demonstrated in Figure

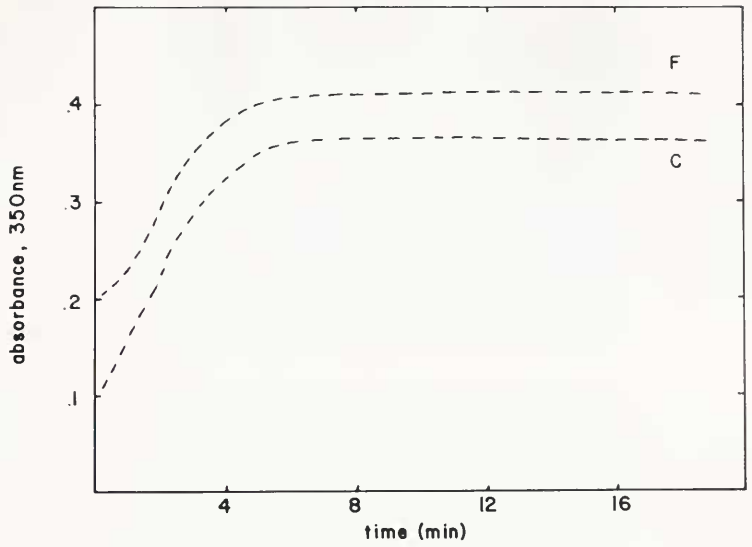

FIGURE 3. Kinetics of assembly of a control (C) and DTAF labeled (F) sample of phosphocellulose purified tubulin dimers, each at 3.2 mg/ml. For this experiment, the fluorescent protein was prepared using a 2:1 molar excess of fluorochrome to protein. Assembly was monitored at 37°C using a temperature controlled cuvette holder. Apparent absorbance at 350 nm has been plotted as a function of time; the initial A_{350} values have been offset along the ordinate using the controls on the spectrophotometer. As in Figure 2, the total change in A_{350} is nearly identical for each sample, as is the initial rate of assembly.

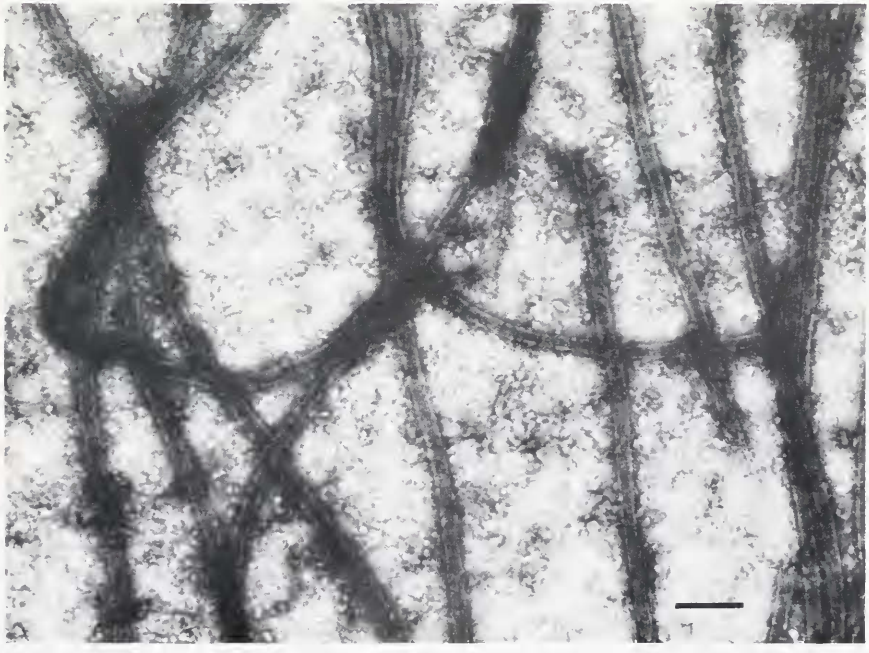

FIGURE 4. Negative stain electron micrograph of assembled fluorescent microtubules. The MTs appear morphologically normal. Bar = 100 nm.

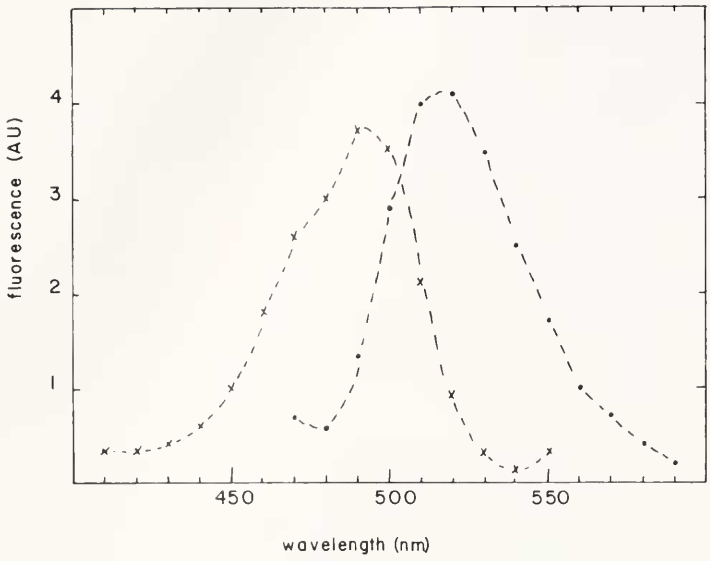

FIGURE 5. Excitation/emission spectra of DTAF labeled microtubule proteins. The excitation maximum (--×--×--) is 495 nm and the emission maximum (--●--●--) is 520 nm. These values are the same whether intact MTs or depolymerized MT proteins are used for the determination. AU = arbitrary units.

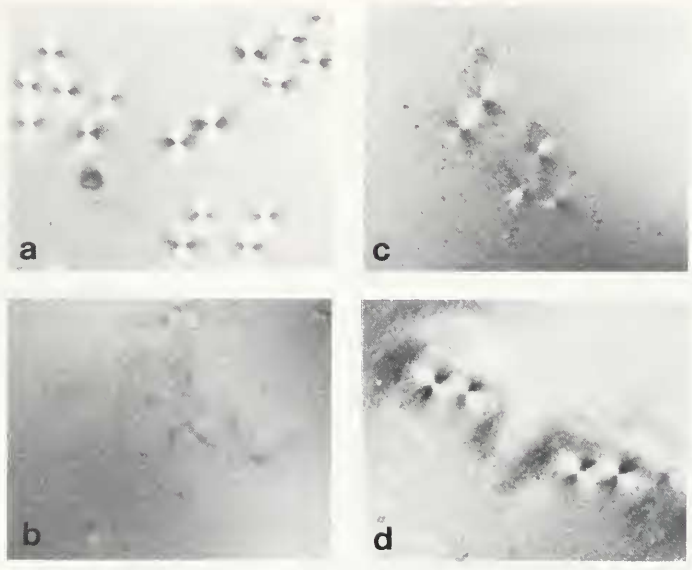

FIGURE 6. Polarized light micrographs demonstrating the effect of fluorescent microtubule proteins on the birefringence of isolated meiotic spindles from the surf clam *Spisula solidissima*. (a) Polarized light micrograph of the spindles immediately after isolation. (b) The same preparation of spindles after centrifugation and resuspension in PM buffer at 4°C. When resuspended in PM buffer containing (c) 0.8 mg/ml and (d) 3.8 mg/ml fluorescent microtubule proteins, followed by incubation at 37°C, BR is observed in proportion to the concentration of fluorescent proteins present.

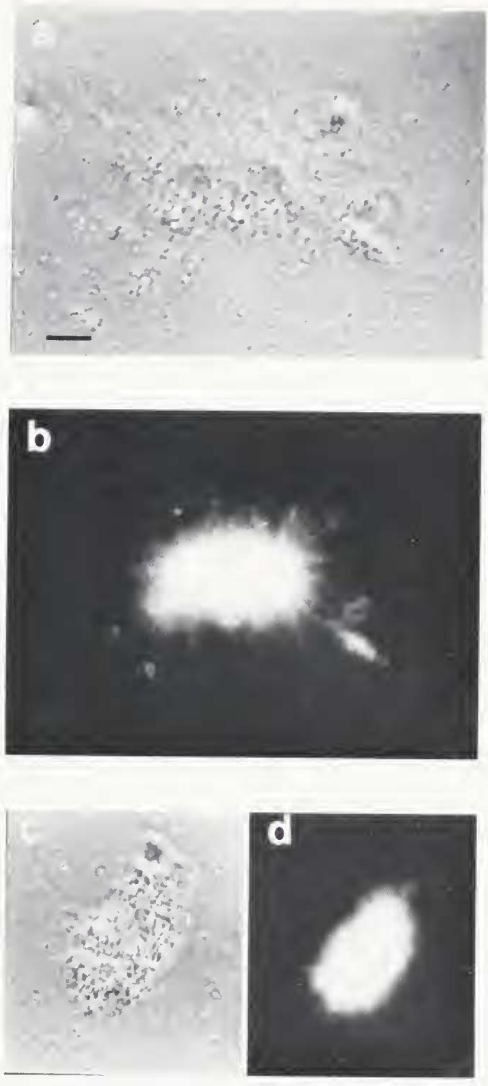

FIGURE 7. Phase contrast (a, c) and fluorescence (b, d) micrographs of *Spisula solidissima* meiotic spindles. (a, b) The spindles were incubated at 37°C with DTAF labeled microtubule proteins at 3.8 mg/ml. Note the distinct, linear fluorescence. (c, d) 3×10^{-3} *M* calcium has been added to the fluorescent spindles; note the loss of linear fluorescence (d) and the alteration in spindle morphology in phase contrast (c). Bar = 20 μm.

1. Moreover, the fluorescent proteins retain their native characteristics with respect to several criteria. For example, the modified proteins assemble with kinetics indistinguishable from controls and appear morphologically normal when examined in negatively stained preparations (see Figs. 2–4). In addition, the fluorescent microtubule protein is capable of assembly in association with isolated spindles *in vitro* and can be visualized and photographed using conventional fluorescence microscopy (Fig. 7).

The resulting stoichiometry for fluorescent tubulin reported here (*ca.* 0.1 mole/mole tubulin) is less than the 0.5 mole fluorochrome: mole tubulin dimer previously reported for tubulin modified with DTAF (Keith *et al.,* 1981). Preliminary experiments, performed using the labeling conditions described by Keith *et al.* (1981), resulted in very low yields of protein that had poor assembly characteristics. Thus, modifications of the Keith *et al.* (1981) labeling procedure were made (see Results). This modified procedure yields tubulin which retains its native characteristics, forms distinctly fluorescent polymers (see Fig. 7), and is therefore suitable for use as a probe of MT assembly *in vitro* and *in vivo.* However, it should be pointed out that much of the tubulin is denatured after exposure to even the modified labeling conditions reported here; thus the DTAF labeled tubulin can only be prepared with a maximum yield of approximately 5.0%.

The labeling procedure described here is a convenient method for preparation of fluorochrome labeled tubulin and MAPs. The tubulin retains its native characteristics and is suitable for use in the technique of fluorescent analog cytochemistry (Wang *et al.,* 1982). As demonstrated by Keith *et al.* (1981), purified, DTAF-labeled tubulin dimers or DTAF-labeled MT proteins compete quantitatively with unlabeled proteins for assembly into polymer. While such an *in vitro* competition assay has not been repeated here, microinjection of DTAF labeled tubulin into living cells demonstrates that this modified tubulin competes successfully with a large excess of tubulin for assembly into polymer (Wadsworth and Sloboda, 1983). Experiments in progress also indicate that the DTAF labeled MAPs retain the ability to bind to MTs *in vivo,* and may be useful probes for analyzing MAP function in living cells.

Acknowledgments

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